UNIVERSITI PUTRA MALAYSIA

GENETIC VARIATION AND ANTIVITY OF NDROGRAPHIS PANICULATA GERMPLASM FROM MALAYSIA

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GENETIC VARIATION AND ANTICANCER ACTIVITY OF *NDROGRAPHIS PANICULATA* GERMPLASM FROM MALAYSIA

By

JEBRIL ALI ABDALLA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Agricultural Science

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DEDICATION

I would like to dedicate this dissertation to honor my parents for their extraordinary contribution in my life. Last but not least, a great appreciation goes to my brothers, my wife and my son for their love.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Agricultural Science

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Chairman: Associate Professor Mohd Said Saad, PhD
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Andrographis paniculata (Acanthaceae) is a medicinal herb endowed with curative properties against a variety of ailments including cold, fever, liver diseases, diabetes and cancer. Pharmacological studies have demonstrated that the major compound of the plant, diterpene lactone andrographolide, have *in vitro* antitumour activity against human breast cancer cell lines MCF-7. Twenty-six *A. paniculata* accessions were collected from three states in Peninsula Malaysia (Selangor, Negeri Sembilan and Perak) to study the genetic variations, phytochemical variations and cytotoxicity activity against MCF-7 cells, in order to identify promising accessions.

Genetic variations of 26 *A. paniculata* were investigated using inter simple sequence repeat (ISSR) analysis. Of the 28 primers screened, 17 produced highly reproducible ISSR bands. Using these primers, 123 discernible DNA fragments were generated with 35 (29 %) being polymorphic, indicating low levels of polymorphism at the populations level. At the 0.84 Jaccard's similarity the *A. paniculata* accessions were divided into 7 distinct groups, the genetic clustering was in agreement with the geographical locations / distances.

Dry material of *A. paniculata* was extracted in petroleum ether dichloromethane, methanol, and mixture of dichloromethane/methanol (1:1) for bioactivity testing. The results indicate that the dichloromethane extracts retains the active compounds contributing for the anticancer activity. Dichloromethane extracts significantly inhibits the proliferation of MCF-7 breast cancer cell lines at low concentrations (11.0µg/ml).

Analysis of the contents of active components of *A. paniculata,* namely andrographolide (ANDRO), neoandrographolide (NAG) and 14-deoxy-11, 12-didehydroandrographolide (DDAG), was carried out using high performance liquid chromatography. Accession 11179S was most superior in terms of andrographolide content, whereby it had 1% in dry weight. Moreover, accessions 11269P, 11265P, 11261P and 11284P also showed higher content of ANDRO of 0.84, 0.78, 0.76 and 0.70% respectively in dry weight.

The lowest amounts for the 3 active compounds were found in accession 11169S, with value of 0.11 – 0.25%.

Dichloromethane (DCM) extracts of the 26 accessions were tested for cytotoxic effect on human breast cancer cell lines, MCF-7, using a microculture tetrazolium (MTT) assay. The accessions had similar cytotoxic activity, determined by the 50% inhibitory concentrations (IC_{50}). Accession 11276P had the lowest IC_{50} value of 2.2 µg/ml, and the highest IC_{50} value of 6.6 µg/ml was shown by accession 11212NS.

In correlation study, the IC_{50} values were negatively correlated with ANDRO ($P \leq 0.05$) and NAG ($P \leq 0.01$), which indicates the choice of accessions with high contents of ANDRO and NAG, results in good activity against MCF-7 human breast cancer cell lines. Also heritability for phytochemicals ANDRO, NAG and DDAG in the present study confirms that these compounds are mainly under genetic control in *A. paniculata* germplasm. Accessions 11179S, 11269P, 11265P, 11261P and 11284P are considered to be the most valuable accessions with high potential for anticancer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Pertanian

VARIASI GENETIK DAN KESAN ANTIKANSER GERMPLASMA *ANDROGRAPHIS PANICULATA* DI MALAYSIA

Oleh

JEBRIL ALI ABDALLA

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Fakulti: Pertanian

Andrographis paniculata Nees merupakan tumbuhan herba yang berupaya untuk merawat pelbagai jenis penyakit seperti selsema, deman, penyakit hepar, diabetes dan kanser. Kajian farmakologi menunjukkan sebatian utama yang terkandung dalam tumbuhan ini ialah lakton diterpena andrographolide yang mempunyai aktiviti antikanser terhadap sel kanser payudara manusia, iaitu MCF-7 secara *in-vitro*. Dua puluh enam aksersi germplasma *A. paniculata* telah dikumpul dari tiga negeri di Semenanjung Malaysia (Selangor, Negeri Sembilan, Perak) untuk kajian variasi genetik, variasi fitokimia dan aktiviti sitotoksiknya terhadap sel MCF-7 dan seterusnya menentukan aksersi yang bermanfaat.

Variasi genetik bagi 26 aksersi *A. paniculata* tersebut dikaji dengan menggunakan primer 'Inter Simple Sequences Repeat' (ISSR). Daripada 28 primer yang dikaji, 17 primer menghasilkan jalur ISSR yang dapat diulangi. Melalui primer ini, 123 pecahan DNA dihasilkan dengan 35 (29%) adalah polimorfik dan ini menunjukkan polimorfisme yang rendah. Persamaan Jaccard pada 0.84 menunjukkan aksersi *A. paniculata* digolongkan kepada 7 kumpulan dengan jelas, dimana pengelompokan genetik adalah sejajar dengan kedudukan geografi.

Bahan kering diekstrak daripada *A. paniculata* dengan menggunakan eter petroleum, diklorometana, metanol dan campuran diklorometana/metanol (1:1). Kajian menunjukkan ekstrak diklorometana mengekalkan sebatian aktif yang memaparkan aktiviti antikanser. Ekstrak diklorometana merencatkan proliferasi sel MCF-7 yang signifikan pada kepekatan rendah. Selain itu, aksersi 11269P, 11265P, 11261P dan 11284P juga mempunyai kandungan ANDRO yang tinggi, iaitu masing-masing sebanyak 0.84, 0.78, 0.76 dan 0.70% berasaskan berat kering. Kandungan ketiga-tiga sebatian aktif yang terendah dilaporkan pada aksersi 11169S dengan nilai 0.11-0.25%.

Ekstrak diklorometana (DCM) untuk 26 aksersi tersebut diuji untuk kesan sitotoksisiti terhadap sel kanser payudara, MCF-7 dengan menggunakan ujian mikcrokultur tetrazolium. Aktiviti sitotoksisiti ditentukan berdasarkan nilai kepekatan perencatan 50% (IC_{50}). Aksersi 11276P mempunyai nilai IC_{50}

terendah iaitu 2.2 μg/ml, manakala aksersi 11212NS mempamerkan nilai IC_{50} yang tertinggi iaitu 6.6 μg/ml.

Analisa kolerasi menunjukan nilai IC_{50} adalah berkorelasi secara songsang dengan kandungan ANDRO ($P \leq 0.05$) dan NAG ($P \leq 0.01$), dimana pilihan aksersi yang mempunyai kandungan ANDRO dan NAG yang tinggi akan memberi aktiviti terhadap sel kanser payudara manusia, MCF-7. Dalam kajian ini, variasi kandungan fitokimia ANDRO, NAG dan DDAG adalah dibawah kawalan genetik. Aksersi 11179S, 11269P, 11265P, 11261P dan 11284P merupakan aksersi yang paling bermanfaat dan berpotensi dalam perubatan antikanser.

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I certify that an Examination Committee met on 18th October 2005 to conduct the final examination of Jebril Ali Abdalla Mohamad on his Master of Agricultural Science thesis entitled "Genetic Variation and Anticancer Activity of *Andrographis paniculata* Germplasm from Malaysia" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Agriculture Science. The members of the Supervisory Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted to any other degree at UPM or other institutions.

JEBRIL ALI ABDALLA MOHAMAD

Date: 8/10/05

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ANDRO	Andrographolide
CDK 4	Cyclin-dependent kinase 4
CRDD	Cancer Research and Drug Discovery
cpDNA	Chloroplast deoxyribonucleic acid
CV	Coefficient of variations
DCM	Dichloromethane
DDAG	14-deoxy-11, 12-didehydroandrographolide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dntp	Deoxynucleoside triphosphate
DTH	Delayed type hypersensitivity
EDTA	Ethylene-diamine-tetra acetic acid
EtBr	Ethidium bromide
FCS	Foetal calf serum
H^2	Heritability
HHT	Homoharringtonin
HIFCS	Heat inactivated foetal calf serum
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IC_{50}	50% inhibitory concentration
ISSR	Inter-simple sequence repeat
KB	Human epidermoid carcinoma
MCF-7wt	Breast cancer cell lines (hormone-dependant)
MDA-MB-231	Breast cancer cell lines (hormone-independent)
MeOH	Methanol
MSE	Mean square for error
MSG	Mean square for genotypes
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide

NAG	Neoandrographolide
NCI	National Cancer Institute
NTSYS-PC	Numerical Taxonomy and Multivariate Analysis System
OD	Optical density
PCR	Polymerase chain reaction
PCR Buffer	[100mM Tris-HCl (pH 9.0) at 25°C; 500mM KCl; 1% Triton X 100]
PE	Petroleum ether
R	Number of replications
RAMP	Random amplified microsatellite polymorphisms
RAPD	Random amplified polymorphic DNA
RCBD	Randomized Complete Block Design
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
RPMI 1640	Roswell Park Memorial Institute – 1640
SAHN	Sequential, Aggtomerative, Hierarchical and Nested clustering
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rats
SIMQUAL	Similarity for Qualitative Data
SRBC	Sheep red blood cells
SSR	Simple Sequences Repeat
TBE	[89 mM Tris-base, (pH 8.3); 89 mM Boric acid; 2 mM EDTA]
tBHP	Tort-butyl hydroperoxide
TE	Tris-EDTA buffer
t_R	Retention time
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
URI	Upper respiratory tract infection
USA	United states of America
UV	Ultraviolet
$\sigma^2 e$	Environmental variance

$\sigma^2 g$	Genotypic variance
$\sigma^2 p$	Phenotypic variance

CHAPTER 1

INTRODUCTION

Throughout the ages, humans have been relying on plants for medicine. Such plants or parts of the plant are known as herbs (Ralph 1996). Among the many medicinal plants, *Andrographis paniculata* (Nees) from the family of Acanthaceae, known commonly as King of Bitters, has been identified to posses a great deal of medicinal values and traditionally has been used in treating various illnesses and disorders (Ong and Nordiana 1999).

Andrographis paniculata is believed to be originated from India and was introduced into Malaysia by the migration of Indian labours. It is known locally in most parts of Malaysia as 'Hempedu Bumi' although several other names are being used by the local people from different areas of the country. It grows as a wild plant in the open fields but at times planted specially for medicinal purposes (Zhang and Tan 1997).

Chemical investigations of *A. paniculata* began as early as 1911 when Gorter (1911) isolated a bitter constituent from its leaves. The compound was trivially named andrographolide. Cava *et al.* (1965) elucidated the complete structure of andrographolide and found that it was a diterpene lactone.

The major ones are andrographolide (ANDRO), neoandrographolide (NAG) and 14-deoxy-11, 12-didehydroandrographolide (DDAG). The main component andrographolide, an unsaturated γ-lactone has been reported to have anticancer activity (Siripong *et al.* 1992).

Despite its enormous medicinal and economic importance, attempts to cultivate *A. paniculata* have seldom been undertaken in Malaysia, in particular, and in most parts of the world. It should be noted that even basic biological data are lacking for *A. paniculata* as also the case with many other medicinal plants. It is needless to say that in the wilderness of nature, the populations of a sexually reproduced species like *A. paniculata* are presumably so varied, that selection of high biomass and product yielding genotypes is distinctly possible and holds much promise for up grading into cultivars.

The involvement of *A. paniculata* in research work and breeding programs for genetic improvement has not been fully realized until recently and also studies on the germplasm of the species are very much lacking. Information on germplasm variations is very important for genetic improvement of the plant. Recent work in our laboratory has shown moderate variation of the quantitative morphological characters within the Malaysian *A. paniculata* germplasm (Melaku 2003).

Several studies has been conducted to evaluate and test the medicinal properties of *A. paniculata*. However genetic variations of the species particularly for the effects on certain illness have been a subject of little investigation (Sabu *et al.* 2001).

Thus, the present study was undertaken to analyse the genetic variations at molecular level and anticancer activity of *A. paniculata* germplasm in Malaysia. The specific objectives were;

1- To study genetic variations of *A. paniculata* germplasm in Malaysia as revealed by Inter–Simple Sequence Repeats (ISSR) markers.

2- To determine Phytochemical variation of the active components of *A. paniculata* namely andrographolide (ANDRO), neoandrographolide (NAG) and 14-deoxy-11, 12-didehydroandrographolide (DDAG).

3- To determine the variation in cytotoxic effect of *A. paniculata* germplasm in Malaysia.